Gene Expression Assay Performance Guaranteed With the TaqMan Assays QPCR Guarantee Program

Real-time PCR for the quantification of gene expression

Real-time or quantitative PCR (qPCR) is one of the most powerful and sensitive techniques available for gene expression analysis. It is used for a broad range of applications, including quantification of gene expression, measuring RNA interference, biomarker discovery, pathogen detection, and drug target validation. When studying gene expression with qPCR, scientists usually investigate changes—increases or decreases—in the quantity of particular gene products or a set of gene products. Investigations typically evaluate gene response to biological conditions such as disease states, exposure to pathogens or chemical compounds, the organ or tissue location, or cell cycle or differentiation status.

Real-time PCR for the quantification of gene expression using the 5' nuclease assay with Applied Biosystems™ TaqMan™ probes has become a standard method in basic and clinical research. The technique is economical, has relatively high throughput, and provides quantitative data. Thermo Fisher Scientific offers a complete suite of Applied Biosystems™ products for the entire qPCR gene expression analysis workflow—from sample prep to reverse transcription and thermal cycling to data analysis. These reagents and instruments enable users to meet the highest standards for performing qPCR experiments. Applied Biosystems™ TaqMan™ Gene Expression Assays (PCR primer and TaqMan probe sets) are tested and optimized for specificity, reproducibility, linear dynamic range, sensitivity, and efficiency in gene expression analyses. These requirements help enable you to perform well-executed qPCR experiments without having to independently test each parameter. In addition, we provide the relevant information on targets and oligonucleotides, as well as protocols required for publication, as is consistent with the MIQE guidelines that describe the minimal elements necessary for publication of real-time qPCR data [1].

The TaqMan Assays QPCR Guarantee

Whether testing a large or small number of targets against a few or hundreds of samples, having confidence in the data you produce is paramount. Thermo Fisher Scientific has developed the TaqMan Assays QPCR Guarantee to provide you with confidence in the data you generate with a predesigned Applied Biosystems™ TaqMan™ Assay. Thermo Fisher guarantees that Applied Biosystems™ TaqMan™ Gene Expression Assays will perform to your satisfaction. If you are not satisfied with the performance of a TaqMan Assay, we'll replace it at no cost or give you a credit for the purchase price of such assays.

What to look for in assay performance

Assay performance depends on a number of factors, including experimental design, the quality of the sample, the reagents and thermal cycling conditions used in the qPCR reaction, and the instrument used for thermal cycling. We measure TaqMan Gene Expression Assay performance using the criteria below. Details on how we measured performance of our assays can be found in the Appendix.

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Specificity

- Assays will amplify the intended target at least 10 C_t values earlier than the gene with the closest sequence homology.
- Assays run in a no template control (NTC) reaction will not produce detectable amplification signal (C_t >38).

Reproducibility

- When different lots of an assay are used with the same sample input and master mix and are run on the same sample plate, the resulting difference in mean C_t values of the two lots will be ≤0.5.

Linear dynamic range and sensitivity

- Assays will provide linear qPCR results over a seven-log range of input template using eight 10-fold dilutions (when C_t is plotted against $\log_{10}$ input template quantity, R^2 value ≥0.98).
- Every assay will detect ≥10 copies of target with a C_t value that is statistically different (*P* value <0.05) than that of the NTC.

Efficiency

- Every assay will exhibit 100% ±10% amplification efficiency when tested in reactions over five orders of magnitude of input template.

Specificity

The TaqMan Gene Expression Assay design pipeline helps ensure high target specificity through the use of up-to-date transcript sequence data, robust primer design algorithms, and extensive bioinformatics tools and processes. Specificity of assays for their intended target is critically important; it means that if a detection signal is seen in a sample, you can be confident that the sample contains your target of interest.

Most TaqMan Gene Expression Assays are designed using transcript sequences from the NCBI Reference Sequence (RefSeq) Project database [2], which is the most highly curated set of non-redundant transcripts in the public domain. It is also the most stable set of sequences available. Each transcript in the RefSeq database has undergone provisional or manual curation, and the majority of transcript sequences were derived from cDNA clones, providing good evidence that they are expressed sequences.

The design pipeline starts with this up-to-date sequence information, then the algorithm masks ambiguous sequences, SNPs, and repeats. Next, assay candidates with probes spanning exon–exon junctions are preferentially selected, so that the assay specifically amplifies the intended transcript(s)—targeting cDNA rather than genomic sequence. Assays are then further refined and optimized, based on thermodynamic and chemical properties: optimal T_m, GC content, secondary structure, amplicon size, and minimal primer-dimer formation. Finally, a three part *in silico* quality control process implements a complex scoring matrix to select assays with the highest transcript specificity, even for members of gene families with high sequence homology.

The outcome of this carefully engineered assay design pipeline is that Applied Biosystems TaqMan Gene Expression Assays detect only their intended target. NTC reactions yield a C_t >38, and reactions containing target amplify the intended target at least 10 C_t values earlier than the next closely related homolog. In addition, we are committed to continual improvement in our design pipeline, including case-by-case analysis for difficult targets such as transcribed pseudogenes [3].

Linking assays to transcripts

During the process of designing an assay, a large number of BLAST searches in the RefSeq and RNA GenBank™ databases are performed. Most assays are found to align with multiple transcript accession IDs. This information is displayed in the TaqMan Gene Expression Assay online ordering system, so that researchers can easily see all the accession IDs with 100% identity to the assay probe and primers, as well as amplicon size, and the assay location on each detected transcript. For clarity and traceability, TaqMan Assay IDs are always linked to the same primer and probe sequence.

Remapping

Because public transcript and genome databases change over time as new sequence information and transcripts are deposited, we use BLAST searches to remap our assays to the updated information every one to two years (using RefSeq and the latest genome assemblies). This keeps the assay alignment information on the Assay Details page current; identifying every known transcript that a particular assay can amplify (with 100% identity to

the assay probe and primers). Note that assays and their associated assay IDs are not changed with these updates; however, the annotation or sequence (transcript or genome) that assays map to may change. For example, assay Hs00364424_m1 was designed in 2004 to the RefSeq NM_033627 (gene symbol TREX1). When the assay was remapped, it was found to match with 100% identity to NM_130384.1, GeneID: 84126 (ATRIP). NCBI had permanently obsoleted NM_033627. Thus, based on the updated annotation, assay Hs00364424_m1 does not map to TREX1: NM_016381. Although the assay annotation has changed, the assay ID and assay sequence have not.

Updating available assays based on new information

Because TaqMan Gene Expression Assays are evaluated for annotation updates and the latest genome information, it is occasionally necessary to remove an assay from our collection because it no longer meets our quality criteria. This may be due to a new SNP that is found in the sequence that binds the primers/probe of the assay, or an assay that becomes nonspecific with the new NCBI annotations at the gene transcript or genome level. Assays may also fail during the manufacturing and functional testing processes. When we obsolete an assay from our catalog we will recommend another assay that is specific for that transcript. Alternatively, we provide mechanisms for the customer to continue to receive the assay through an alternative ordering system (Quick Order) or as a Custom TaqMan Gene Expression Assay [6].

Reproducibility through manufacturing quality

TaqMan Gene Expression Assays are made using validated manufacturing processes that include stringent manufacturing QC criteria: assay identity is confirmed by mass spectrometry and assay concentration is determined using quantitative spectrophotometry. TaqMan Assays are formulated ready-to-use right out of the tube, eliminating the need to optimize primer and probe concentrations. Final 1X reaction concentrations are 250 nM TaqMan probe and 900 nM of each PCR primer. Assays are designed to be run with recommended Applied Biosystems™ TaqMan™ Master Mixes, using universal thermal cycling conditions.

Because researchers often use multiple lots of assays to complete a research study, we validated our manufacturing process to help minimize lot-to-lot variability. We tested the functional performance of eight randomly selected TaqMan Gene Expression Assays across multiple manufacturing lots using the recommended reagent and cycling conditions. Established QC specifications were used as criteria to evaluate the performance of each assay over 24 lots for each of the assays. All assays passed NTC testing to rule out contamination (C_t >38). All assay lots tested with synthetic DNA had a range of C_t values that were consistently within the specification of ±0.5 C_t (Figure 1). These data show that the mean difference will be no more than 0.5 C_t between two manufacturing lots when run in quadruplicate on the same plate for the same sample.

Linear dynamic range and sensitivity

The dynamic range of an assay can be defined as the relationship of signal (C_t value) between the highest- and lowest-diluted samples (C_t vs. $\log_{10}$ of input) that generates linear data with high correlation. Using either a sample with highly abundant target, a cloned sequence, an artificial template, or an amplicon as the reaction template, TaqMan Gene Expression Assays have a dynamic range of at least seven logs with R^2 >0.98. The reliability of C_t measurement decreases as the copy number decreases to one copy due to Poisson statistics, thus the functional dynamic range actually begins at concentrations greater than ~10 copies per reaction (Figure 2).

Here we define assay sensitivity as the number of template copies an assay can detect above an NTC background on a specified platform. For TaqMan Gene Expression Assays, we guarantee that the C_t of a 10-copy sample (C_t ~35) will be less than the C_t of the NTC with a *P* value <0.05.

Efficiency

Amplification efficiency is a major concern for any real-time PCR–based assay product because reliable qPCR results depend upon a doubling of PCR product at every PCR cycle. We have performed extensive validation to demonstrate the nearly perfect amplification efficiency of TaqMan Gene Expression Assays. A set of more than 750 TaqMan Gene Expression Assays were selected to represent a wide variety of challenges for efficient amplification and plot

including a wide range of GC content, amplicon lengths, and secondary structure tendencies. The assays exhibited amplification efficiency values ranging from 90–110%, and the distribution approximated a normal distribution with a mean of 98.73% (Figure 3). In this study we determined that a dilution series of at least five logs was necessary to accurately calculate assay efficiency [4]. Efficiency estimates can vary significantly if a rigorous measurement protocol is not followed:

- Measurements must be obtained over a broad dilution range (five to six logs) and include replicates to decrease the effects of laboratory errors (i.e., pipetting accuracy).
- Efficiency of a PCR reaction can be affected by substances known to inhibit PCR, such as heme and detergents; users must ensure that cDNA templates are free of PCR inhibitors.

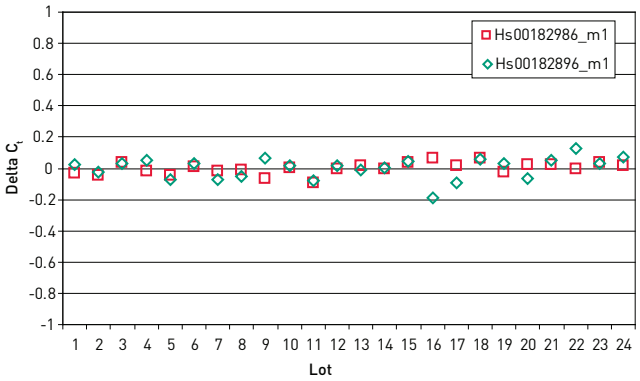

Figure 1. TaqMan Gene Expression Assays exhibit lot-to-lot consistency. Twenty-four lots were sampled for each of eight TaqMan Gene Expression Assays. Each assay was tested in quadruplicate on 384-well plates, thus each lot of each assay was tested four times for a total of 192 lots. Synthetic targets with Applied Biosystems™ TaqMan™ Universal PCR Master Mix were run with each assay to measure C_t values. Analysis was performed with SDS 2.3 software using a manual threshold of 0.2 and an automatic baseline setting. The ΔC_t for each lot prepared for Hs00182986_m1 and Hs00182896_m1 was calculated by subtracting the average C_t of a single lot tested in quadruplicate from the average C_t of all 24 lots. The ΔC_t for each assay lot is shown. Open red square = Hs00182986_m1; open green diamond = Hs00182896_m1.

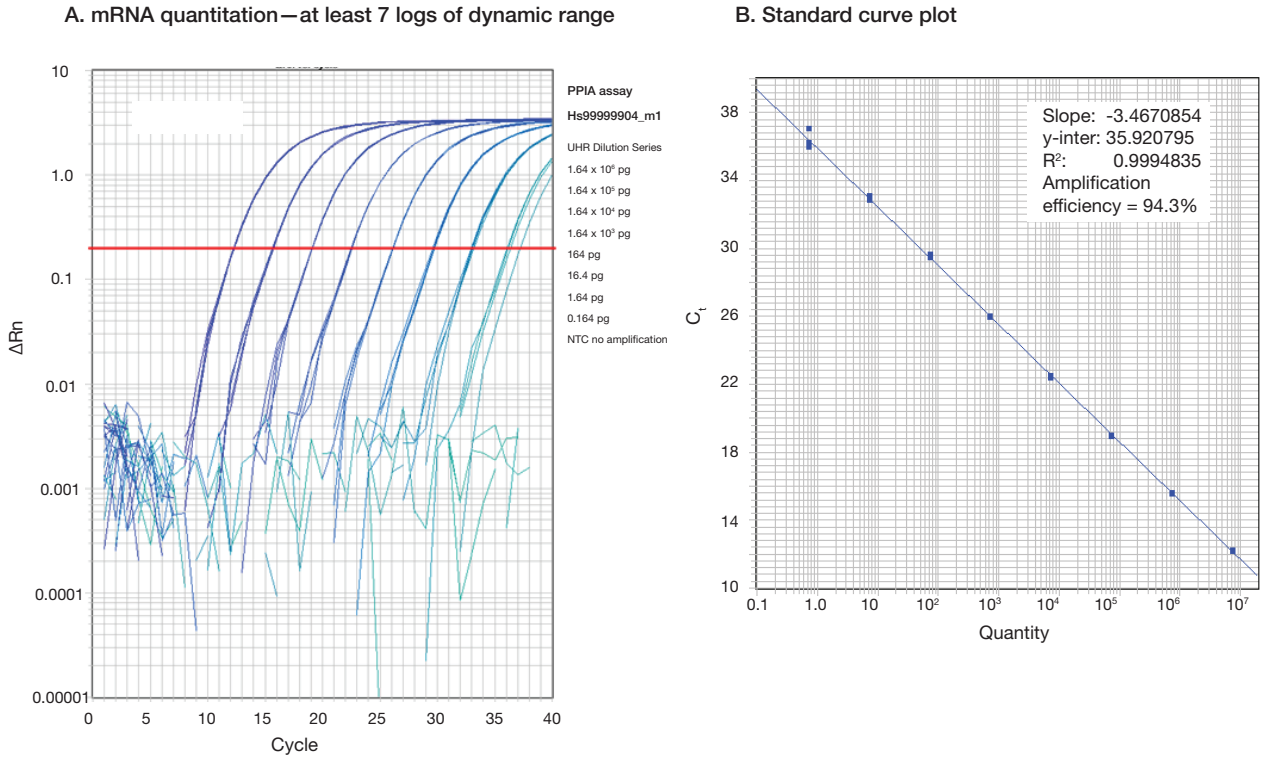

Figure 2. Linear dynamic range of over 7 logs and sensitivity down to 10 copies. (A) Linear dynamic range: amplification plot showing 7 10-fold dilutions of preamplified UHR cDNA run with Hs99999904_m1 (PPIA). Four replicates were run for each dilution point (average standard deviation = 0.1). The calculated R^2 is 0.999; slope = –3.4. **(B)** Sensitivity (limit of detection) is estimated at 10 copies: cDNA at estimated copy number of 107 to 10 copies (1.64 x 10^6–1.64 pg cDNA) were run in parallel with NTC (n=4). Sufficient replicates were run to determine statistical difference (*P* value <0.0004 between NTC and lowest copy number (estimate one copy)).

An assay tested under these conditions will exhibit 100% ±10% amplification efficiency in reactions containing five orders of magnitude of mass amount of input template.

Conclusion

We understand that confidence in your gene expression data is vital, and the criteria described above are important considerations in design of assays and experimental protocols. Through rigorous testing, quality control, and sophisticated software tools, we have designed TaqMan Gene Expression Assays to enable you to obtain robust, reproducible gene expression data.

Learn more

For more information about the Applied Biosystems TaqMan Assays QPCR Guarantee Program, its restrictions, and terms and conditions, visit us online at **thermofisher.com/taqmanguarantee** or contact your local sales representative.

To learn more about ready-made and custom TaqMan Gene Expression Assays, visit us online at **thermofisher.com/allgenes** or contact your local Applied Biosystems sales representative.

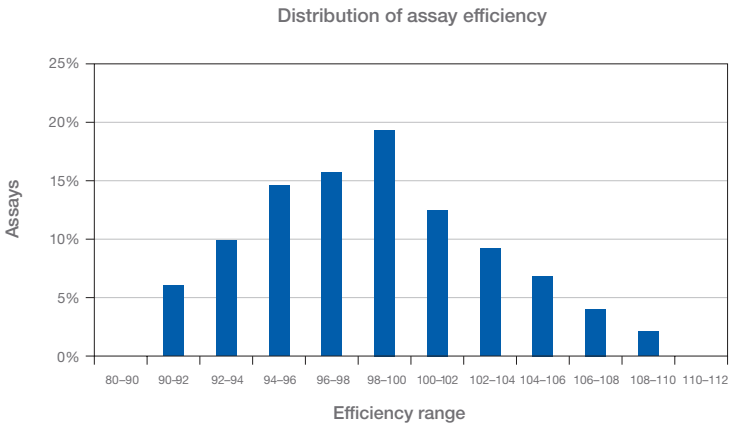

Figure 3. 100% (±10%) Amplification efficiency. The amplification efficiency of over 750 TaqMan Gene Expression Assays representing a wide range of oligonucleotide compositions (for example, base composition, amplicon length, and secondary structural features) was evaluated. The amplification efficiency ranged from 90% to 110% with a mean of 98.73%.

References

1. Bustin SA, Benes V, Garson JA et al. (2009) The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. *Clin Chem* 55(4):611–622.
2. Pruitt KD, Tatusova T, Maglott DR (2007) NCBI reference sequences (RefSeq): a curated non-redundant sequence database of genomes, transcripts and proteins. *Nucleic Acids Res* 35(Database issue):D61-5; http://www.ncbi.nlm.nih.gov/refseq/.
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5. Real-time PCR: Understanding C_t. Applied Biosystems Application Note (can be found online at http://www3.appliedbiosystems.com/cms/groups/mcb_marketing/documents/generaldocuments/cms_053906.pdf).
6. TaqMan Gene Expression Assays Protocol, Part Number: 4333458 (can be found online at: http://www3.appliedbiosystems.com/cms/groups/mcb_support/documents/generaldocuments/cms_041280.pdf).

Appendix

Specificity and assay design

The determination of cDNA specificity relative to genomic DNA is important because RNA samples can be contaminated with gDNA depending on the purification method utilized; however, the potential for this can be minimized by using a good RNA purification method or DNase treatment along with quality control of the prepared RNA samples. For guidelines on sample acquisition, handling, preparation, or QC of nucleic acids, see Bustin et al. [1].

The TaqMan Gene Expression Assay ID

The TaqMan Gene Expression Assay ID suffix provides information on the placement of the probe on the transcript. The suffix is assigned at the time the assay is designed and may change. For example, if the probe position is shifted with an updated genome assembly, a change in the suffix may result. The change will be described in the Important Information link for a given assay. Note that suffix changes have more to do with the target that the assay will detect than the assay performance.

Suffix	Description of suffix (indicates the assay placement)
_m	_m indicates an assay whose probe spans an exon junction and one that will not detect genomic DNA.
_s	_s indicates an assay whose primers and probes are designed within a single exon. Such assays will, by definition, detect genomic DNA.
_g	_g indicates an assay that may detect genomic DNA. The assay primers and probe may also be within a single exon.
_mH _sH _gH	_mH, _sH, or _gH indicates that the assay was designed to a transcript belonging to a gene family with high sequence homology. The assays have been designed to give between 10 C_t and 15 C_t difference between the target gene and the gene with the closest sequence homology. This means that an assay will detect the target transcript with 1,000 to 30,000-fold greater discrimination (sensitivity) than the closest homologous transcript, if they are present at the same copy number in a sample.
_u	_u indicates an assay whose amplicon spans an exon junction and the probe sits completely in one of the spanned exons.
_ft	_ft indicates an assay designed to detect fusion transcripts that result from chromosomal translocation. One primer and the probe are located on one side of the fusion transcript breakpoint.
_at	_at indicates an assay that is designed to detect a specific synthetic RNA transcript with a unique sequence that lacks homology to current automated biological sequences.

Procedures used to test assay performance

Below are descriptions of the procedures and expected results for the performance testing. All qPCR tests described below were performed according to the TaqMan Gene Expression Assays Protocol (Cat. No. 4333458, see reference 6) and:

- The sample and reaction conditions, reagents, and instrument and thermal cycling parameters used were those recommended in the protocol.
- Samples were free of PCR inhibitors and impurities.

How to test for PCR inhibitors in the sample

1. Identify a gene that displays moderate expression in the sample. Serially dilute the sample to obtain five to seven 10-fold dilutions, with the center of the series containing a mid-range quantity of template DNA, having a C_t of 27 to 28. Because this experiment depends on accurate dilutions, set up the experiment so that pipetting steps contain at least 5 µL—use calibrated pipettors. In general, Good PCR Practices should always be followed (see Appendix B in TaqMan Gene Expression Assays Protocol (Cat. No. 4333458, see reference 6).
2. Amplify the diluted samples with a chosen TaqMan Gene Expression Assay, using a minimum of four technical replicates. The assay should be directed to a high expressed target and/or the sample concentration should be adjusted in order to measure ΔC_t values over the dilution range.
3. Use the Standard Curve (Absolute Quantitation) option within the SDS software to plot and analyze the results.

Result:

- The ΔC_t between each point on the curve should average ~3.3.
- Inhibitor in the sample will typically result in a ΔC_t of <3.3 between 10-fold dilutions. The effect of inhibitors is usually highest in the more concentrated samples and reduced in more dilute samples.

How to test for specificity

Assays are designed to amplify the intended target at least 10 C_t values earlier than the gene with the closest homology.

1. To test that an assay amplifies the intended target specifically compared to a homologous sequence, we used individual plasmids or artificial templates that contain the target and homologous sequences.
2. In order to run a test for specificity, verify that the templates are amplified by the assay to be tested.
3. Adjust the concentration (e.g., ng/µL) of the template for the intended target to yield a C_t <30 in real-time PCR; then adjust the homologous sequence template(s) to the same concentration(s).
4. Run the assay with a minimum of four technical replicates on the same plate following the recommended protocol.

Result:
The ΔC_t between the intended target and homologous sequence templates should average >10 C_t.

Assays run in a no template control (NTC) reaction will not produce detectable amplification signal (C_t >38).

1. Set up an NTC reaction mix using the qPCR reagents and the TaqMan Assay being tested, but with molecular biology–grade water in place of sample.
2. Amplify a minimum of four technical replicates.

Result:
NTC reactions should yield C_t values >38 with a proper threshold and baseline setting. Note that it is easier to set an appropriate threshold when there are also "real" amplified samples in the experiment for comparison.

To make sure that the reagents and TaqMan Assay are not contaminated with PCR products, consider running another set of NTC reactions using a second TaqMan Assay.

How to test for manufacturing reproducibility

When different lots of an assay are used with the same sample input and master mix, and are run on the same sample plate, the resulting difference in mean C_t value of the two lots will be ≤0.5.

1. Prepare a reaction mix containing sample and qPCR master mix, except the assay, sufficient for a minimum of four technical replicates for each TaqMan Assay lot to be tested. Include enough sample for each replicate to yield a C_t <30 with a properly set baseline and threshold.
2. Aliquot the mixture for the number of TaqMan Assay lots to be tested, then add the TaqMan Assay to the mixtures. Pipette the technical replicates into individual plate wells, and run the assays.
3. Determine the C_t average for each assay lot.

Result:
The ΔC_t between average Ct_t of each assay lot should be ≤0.5.

How to test for linear dynamic range and sensitivity

Assays will provide linear qPCR results over a seven-log range of input template mass using eight 10-fold dilutions (when C_t is plotted against $\log_{10}$ input template quantity, R^2 value ≥0.98).

Every assay will detect ≥10 copies of target with a C_t value that is statistically different (*P* value <0.05) than that of the NTC.

To test for efficiency, a relatively high concentration of template containing the amplicon for this experiment is needed. The protocol suggests that a PCR product be used (being careful to avoid contamination of the lab with amplified DNA) or a cDNA clone at a high template concentration.

1. Prepare 10-fold serially diluted template to cover seven orders of magnitude (eight dilution points). The most dilute sample should contain ≥10 copies of the amplicon (C_t<35). Prepare enough material for at least four technical replicates per dilution, plus an NTC. As stated above, this experiment depends on accurate dilutions. Pipetting steps should contain at least 5 µL using calibrated pipettors. Refer to Good PCR Practices [in Appendix B in TaqMan Gene Expression Assays Protocol (Cat. No. 4333458, see reference 6).]
2. Plot the C_t versus $\log_{10}$ input template amount. Recommended Applied Biosystems instrument software may be used, or export the C_t values to Microsoft™ Excel™ software and plot the standard curve. [Applied Biosystems™ SDS v2.3 software was used to generate the Standard Curve Plot with slope, y-intercept, and R^2 value.]

Result:
The expected R^2 ≥0.98.

3. Determine the statistical difference between the lowest dilution and the NTC by performing a Student's *t*-test.

Result:
The *P* value should be <0.05, indicating that there is a statistically significant difference between the lowest dilution and the NTC.

How to test for efficiency

Every assay will exhibit 100% ±10% amplification efficiency when tested in reactions over five orders of magnitude of mass amount of input template. (For background on testing amplification efficiency, refer to the Amplification Efficiency of TaqMan Gene Expression Assays application note [4].)

This test is similar to the linear dynamic range test—a relatively high concentration of template containing the amplicon for this experiment, with 10-fold serial dilutions, is needed.

1. Prepare 10-fold serially diluted template to cover at least five orders of magnitude (six dilution points) of input template quantity. Each dilution must contain enough template to generate C_t values <35. Prepare enough material for at least four technical replicates per dilution, plus an NTC. This experiment depends on accurate dilutions. The experiment should be set up so that pipetting steps contain at least 5 µL—calibrated pipettors and Good PCR Practices should be used [6].

2. Plot the C_t versus $\log_{10}$ input template amount and determine the slope of the line that can be drawn through the data points. Recommended Applied Biosystems instrument software may be used, or export the C_t values to Excel and plot the standard curve. Applied Biosystems SDS 2.3 software was used here.

3. Calculate the amplification efficiency of the assay from the slope of the curve.
   Amplification efficiency = 10(-1/slope) – 1.

Result:
Assays should have 100% efficiency (±10%).

Find out more at **thermofisher.com/taqmanguarantee**

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